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DSTL, WO 189/778, 29 May 2009

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FROM

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AUTHORITY

British Embassy notice dtd 30 Nov 1983

MINISTRY OF SUPPLY

DIRECTORATE OF CHEMICAL DEFENCE RESEARCH AND DEVELOPMENT

CHEMICAL DEFENCE EXPERIMENTAL ESTABLISHMENT

A FIELD TEST FOR THE ASSAY OF HUMAN WHOLE BLOOD CHOLINESTERASE

By

D.R. DAVIES AND J.D. NICHOLLS

PORTON TECHNICAL PAPER No. 446

C.D.E.E.
Porton.
Wilts.

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SUMMARY

The field test of Limperos and Ranta for the assay of human whole blood ChE has been modified to facilitate its more extensive use under field conditions. A chart has been constructed which permits adjustments in the conditions of the test to compensate for temperature variation and to relate ChE levels to the degree of poisoning.

(Sgd.) H. Cullumbine,
Supt., Medical Division.

DRD/JDN/SMI.

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Introduction

The level of cholinesterase (ChE) in the blood is a valuable index of the amount of anticholinesterase which has been absorbed into the system and it has in fact been used for this purpose (Metcalf 1951, Davies 1952 and Marchand 1952). Since anticholinesterases are being used extensively in agriculture and for insect vector control - very often far removed from laboratory facilities - the determination of such an index under field conditions would be most useful. The essential requirement of the field test is simplicity. The procedure and equipment required should be simple, the number of reagents required few and the time taken for the performance of the test reduced to a minimum. Finally the test should be sufficiently uncomplicated as to be carried out by non-specialist personnel.

Aldridge and Davies (1952) have discussed the possibility of applying the generally used techniques for ChE assay to field conditions and they came to the conclusion that, for reasons given, they were unsuitable. They did however suggest that the electrometric method of Michel (1949) was satisfactory under certain circumstances. They based their suggestion on the fact that this technique lent itself to the rapid and easy estimation of considerable numbers of samples. Whilst subsequent experience has shown this to be substancially true, there are two major disadvantages. The pH meter is not sufficiently reliable to use in the field; and the glass electrode is most certainly too fragile for this purpose.

Fleisher and Pope (1954) have applied the Hestrin colorimetric method (1949) to the estimation of whole blood ChE. Their method possesses the advantages that it is a colorimetric technique and that single estimations can be completed, including the collection of the blood sample, in twenty-five minutes. Its disadvantages as a field test are (1) the number of tests that can be performed in a given time is relatively few and (2) at least six reagents and an equally large number of manipulative procedures are involved. Furthermore the technique requires the use of a spectrophotometer.

Limperos and Ranta (1953) have recently described a test which they have used under field conditions and which approximates to that which is required. The principle is the same as that of the electrometric method. The differences are (1) that changes in pH are followed by changes in a bromthymol-blue (BTB) solution incorporated in the reaction mixture and (2) the

test is carried out in an unbuffered medium. The various levels of enzyme activity are assessed by the colour of the solution after a fixed interval of twenty minutes and these colours are green, olive green, olive brown and orange. In practice the distinction between the first two and the second two colours is difficult. This is a serious disadvantage since (as will be shown later) this is the important range. A second major disadvantage is that under ordinary conditions of illumination the combination of blood and BTB appears to fluoresce. When this happens colour assessment is almost impossible. Despite these difficulties a relatively slight modification of procedure has been shown to give a workable and satisfactory test. If a fixed colour change is taken (i.e. from green to orange) and the time for this change to occur observed these difficulties can be overcome.

Experimental

Apparatus. Tubes 3 x 3/8 in. (one for each test)
Pipettes 10 ml. graduated in 1.0ml.
5 ml. graduated in 0.5ml.
20 c.mm. haemoglobin pipette.
Rack - to hold ten 3 x 3/8 in. tubes.
Watch - to read accurately in minutes.
Triangular needles - 1 packet.
Thermometer.

Reagents Two solutions only are needed.

Acetylcholine Chloride - Ampoules - Roche product.
0.6% solution. Dissolve the contents of six 0.1 ampoules in 100 ml. of distilled water. This solution can be stored at 20°C. for a period of five or six days without deterioration and provides sufficient solution for 200 tests.

Brom-thymol-Blue (B.D.H.)

(a) stock solution, 0.04 g. of powder is dissolved in 1 ml. of N/20 sodium hydroxide. Approximately 90 ml. of distilled water are added and then sodium hydroxide dropwise until the solution just turns blue. The total volume is made up to 100 ml. The pH of this solution should be 7.7 and if it is possible, since this solution can be made up in the laboratory, it should be checked with a pH meter.

(b) For use, dilute two volumes of stock solution with one volume of distilled water.

Technique

Collect 20 c.mm. of blood in a haemoglobin pipette and wash it out into 1.0 ml. of dilute B.T.B. solution contained in one of the 3 x 3/8 in. tubes. Add 0.5 ml. of acetylcholine chloride solution and note the time of addition. Note the exact time at which the colour of the reaction mixture becomes deep orange. The interval is a measure of the enzyme activity. To facilitate the determination of the end point a standard is easily prepared as follows. Blood and B.T.B. solution in the above proportions are added to a tube and then 0.15 ml. of N/100 acetic acid. The volume is made up to 1.5 ml. (The pH should be 6.7).

Results

If the time interval over which the requisite colour change occurs is determined precisely, then the assessment of whole blood ChE activity correlates closely with determinations made by the electrometric method. This is shown in figures 1 and 2, in which the reciprocal of the time (T) taken to effect the given colour change at temperature (t) is plotted against the activity of the sample in ΔpH/hour, always determined at 25°C. In figure 1 T was determined between 19°C and 22°C. In figure 2 the temperatures were 30°C and 32°C. The close correlation between ΔpH/hr. and the reciprocal of T is obvious from the graphs.

In figure 3 the relative activities of different specimens at varying temperatures have been plotted against the temperature, the activity at 20°C being taken as equivalent to 100. The relative activity increases with temperature in a non-linear fashion from 10°C to 40°C. Above 40°C results become uncertain. At 10°C the relative activity is about 65 whilst at 30°C it is 132.

The information yielded by these two series of experiments permits us to simplify the test for field conditions. If in addition it is possible to relate enzyme levels to zones which will permit clear cut executive action, then a very useful test may be evolved. This latter idea is possible for three critical levels of whole blood ChE can be defined.

(1) The lower level of normal whole blood ChE activity.

(2) The level of hypersensitivity.

(3) The clinical danger level.

These are as follows:-

(1) The Lower level of Normality: There have been very many determinations of normal values of red cell, plasma and whole blood ChE and the limits of variation have been extensively worked out (Callaway et al 1951; Wolfsie and Winter, 1952; Fleisher and Pope, 1954). These are consistent with each other. For the present purpose the values for the whole blood ChE as determined in this laboratory have been used to define the lower limit of normal activity. On a recently determined series the mean value of the whole blood ChE was 133 (ΔpH/hr.) with a standard deviation of 12 (ΔpH/hr.). The lower fiducial limit of normality is therefore 109 (ΔpH/hr.).

(2) The level of hypersensitivity. No symptoms of systemic poisoning are obvious until a very significant reduction of the blood ChE has occurred. Callaway et al (1954) however have shown that when the whole blood ChE of rabbits has, by prior exposure to anticholinesterase, been reduced tohalf the pre-exposure value, there is a significant reduction in the LD50, thus demonstrating that a reduction in the blood ChE level is indicative of an increased sensitivity towards further doses of anticholinesterase. Their data did not permit an extrapolation which would have indicated the exact point at which this increased sensitivity is first obvious. In the absence of such precise data, and bearing in mind the main purpose for which this test is being evolved, we therefore suggest that the level of clinical significance could be set at 50% of the mean normal level, i.e. at a ΔpH/hr. of 67.

(3) The clinical danger level. Davies (1952) noticed that the blood cholinesterase of animals dosed with anticholinesterases by the intravenous, intramuscular or percutaneous routes had to be reduced to at least 20% of normal before the onset of serious clinical symptoms. In this author's experience the critical whole blood cholinesterase level is about 20% of normal, although it does not follow that symptoms automatically appear when this level of blood cholinesterase activity is reached (Fowley et al 1953). The clinical danger level may therefore be set at 20% of the mean normal level, i.e. at ΔpH/hr. of 25 units.

Effect of temperature

The most difficult problem has been to evolve a method of temperature compensation without the use of a constant temperature bath or incubator. This was done as follows. As indicated above, several simultaneous observations were carried out on the same sample at various temperatures, together with electrometric determinations at 25°C. These results were then summarised in a family of straight lines corresponding to each temperature and relating 1000/T and ΔpH/hr. (Figure 4), where T is the time taken for the completion of the colour change.

From these, T was determined for each critical level, at varying temperatures and a new family of curves constructed relating T and temperature. By plotting these on one diagram, as in figure 5, a chart showing various zones has been constructed, each corresponding to the interpretation shown on the right hand side.

The 'modus operandi' of the test is therefore as follows. The temperature of the ambient air is noted. An ordinate on the chart is then drawn corresponding to this temperature, and the time read off at the point at which it cuts each curve.

The tubes are prepared as described earlier (i.e. 1.0 ml. of B.T.B. 20 c.mm. of blood and 0.5 ml. acetylcholine chloride) and then the colours noted at each of those times obtained from the chart (Figure 5). In all the tubes in which the colour has changed to deep orange at the time of inspection the reaction is complete. For example, if the temperature were 23°C, tests with all normal blood samples would be complete in 21 minutes. Blood samples which changed colour in less than 34 minutes but longer than 21 minutes would possess whole blood ChE levels lower than normal and the level would indicate a slight absorption of anticholinesterase. Such levels would however be of little clinical significance. They would on the other hand, indicate indifferent safety procedures or personal carelessness on the part of an individual.

If the time taken is longer than 34 minutes, this indicates that the individual is hypersonsitive, and will come into the red zone. If even after 72 minutes the test is not complete at least 80% inhibition is indicated implying danger of clinical symptoms.

It shoudl be emphasised that an exact colour match with the standard is seldom necessary since the criterion at the critical time is whether the colour change is complete at that time.

One other point with reference to temperature needs to be discussed. The test may, in extreme circumstances (i.e. at 10°C), take 100 minutes to

complete, and if no constant temperature bath is to be used, it is rather important to know what temperature fluctuation may be permitted during the period of the test itself. The experiments on which the relationship between the field test and the electrometric method (shown in Figure 1) were based, were carried out at 19°, 20° and 22°C; hence a variation of 3°C during the period of the test would appear to be permissible. Lowrey et al (1954) confirms this suggestion for they state that to measure cholinesterase to within 3 per cent the permissible temperature range is 3°C. This is however a point that ought to be checked under a wide variety of conditions, or at least kept in mind during the period of the test.

A series of experiments was carried out at different temperatures to check the chart which has been produced. The procedure was exactly as described. 48 samples of blood were prepared by diluting normal blood with inactivated blood. 30 samples (obtained from 8 individuals) were examined on different occasions in the climatic chamber at 31°C and 85% relative humidity. A further 18 samples (from 4 individuals) were tested in a constant temperature bath at 31°C. The results are shown in Table 1.

Table 1

The confirmation of Reaction Times at 31°C.

pH/hr. values and reaction times grouped according to the clinical zones described in the chart (figure 5)

Normal Range		Range of Slight Absorption		Hypersensitive Range	
ΔpH/hr. 109 + T_{31} <18 mins.		pH/hr. 109-67 T_{31} 18-29 mins.		pH/hr. 67-25 T_{31} 29-67 mins.	
130	15	108	20	66	31
126	15	104	20	63	32
146	14	103	21	59	42
132	15	103	20	58	39
123	15	102	20	56	40
140	14	100	22	55	33
129	15	100	22	53	40
148	15	96	22	52	40
136	16	95	23	49	35
133	15	88	23	36	47
127	15	87	23	36	50
146	15	85	26	33	50
116	19	81	25	32	52
116	19	78	27		
110	18	76	27		
		75	27		
		75	28		
		73	28		
		67	27		

Of the 48 samples so tested 45 conform precisely to the pattern described in the text. Of the discrepant samples those with pH/hr. 116, $T_{31}° = 19$ minutes are only just outside the expected zone. A sample of ΔpH/116 should have had a reaction time of 18 minutes or less.

Summary

The field test of Limperos and Ranta for the assay of human whole blood ChE has been modified to facilitate its more extensive use under field conditions. A chart has been constructed which permits adjustments in the conditions of the test to compensate for temperature variation and to relate ChE levels to the degree of poisoning.

(Sgd.) H. Cullumbine,
Supt., Medical Division.

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FIG. 1.

THE RELATIONSHIP BETWEEN A_T AND ΔpH.
A_T VALUES WERE DETERMINED BETWEEN 19° AND 22°C.
ΔpH WAS DETERMINED AT 25°C.

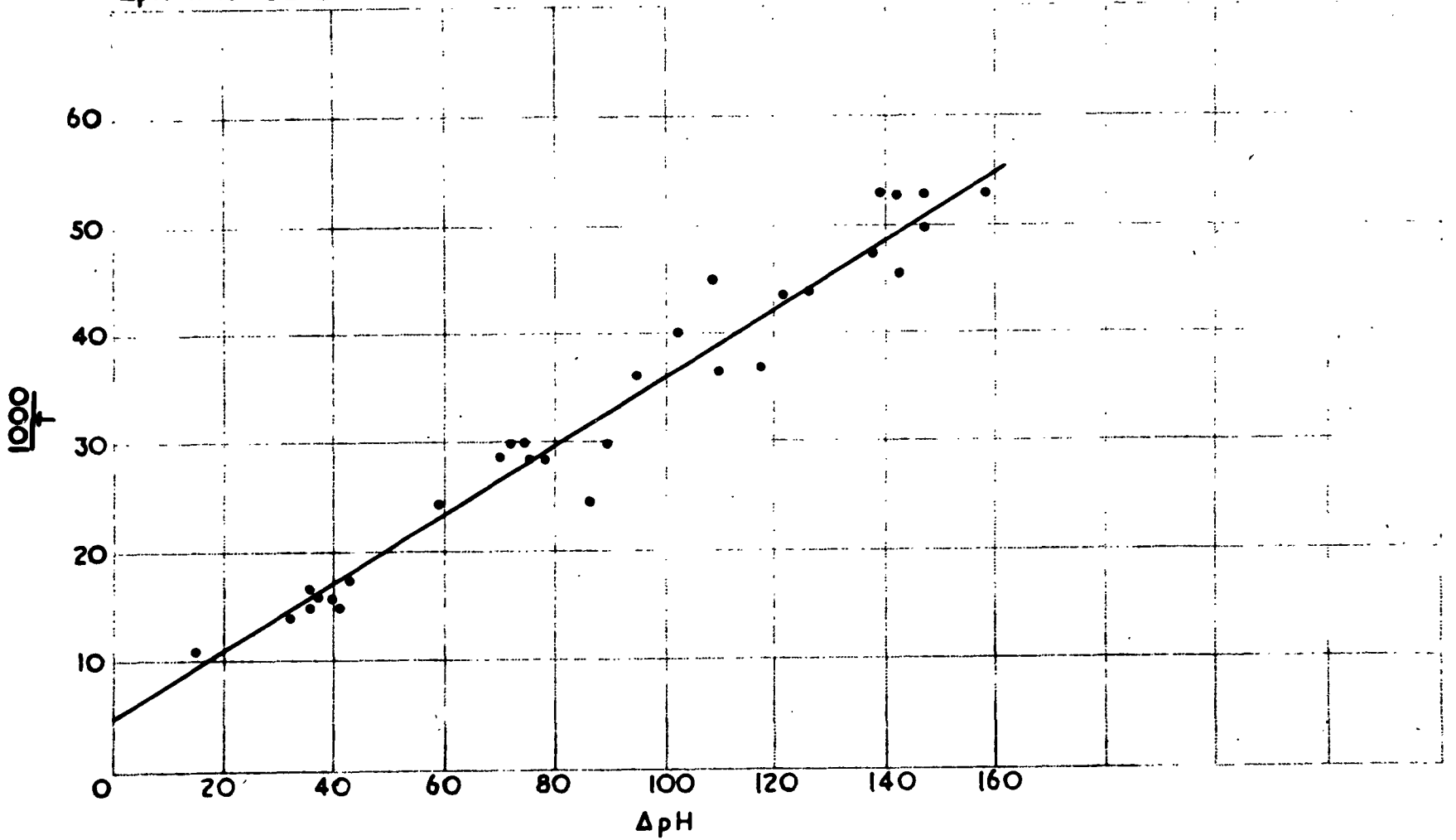
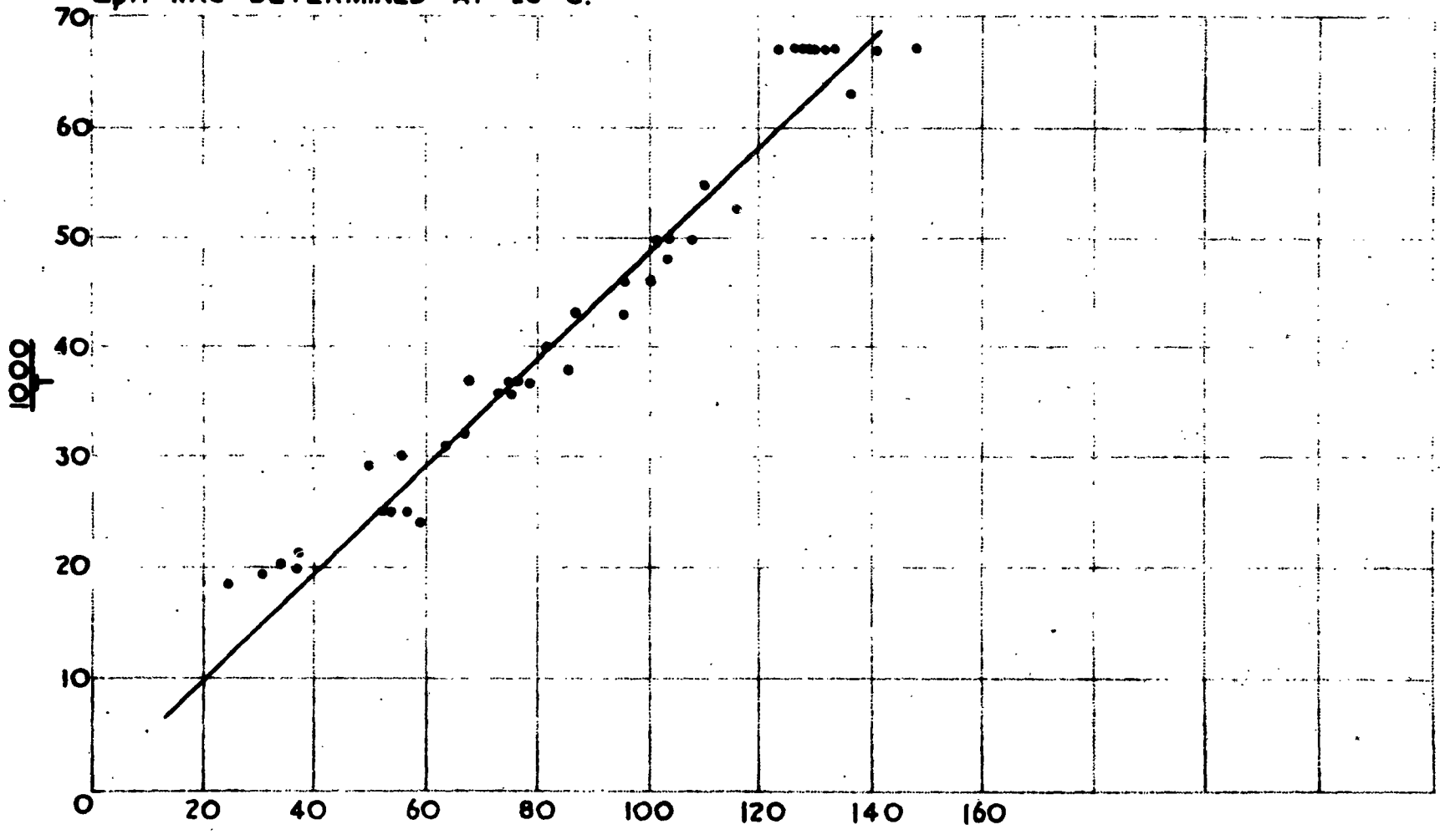


FIG. 2.

THE RELATIONSHIP BETWEEN A_T AND ΔpH.
A_T VALUES WERE DETERMINED BETWEEN 30°- 32°C.
ΔpH WAS DETERMINED AT 25° C.



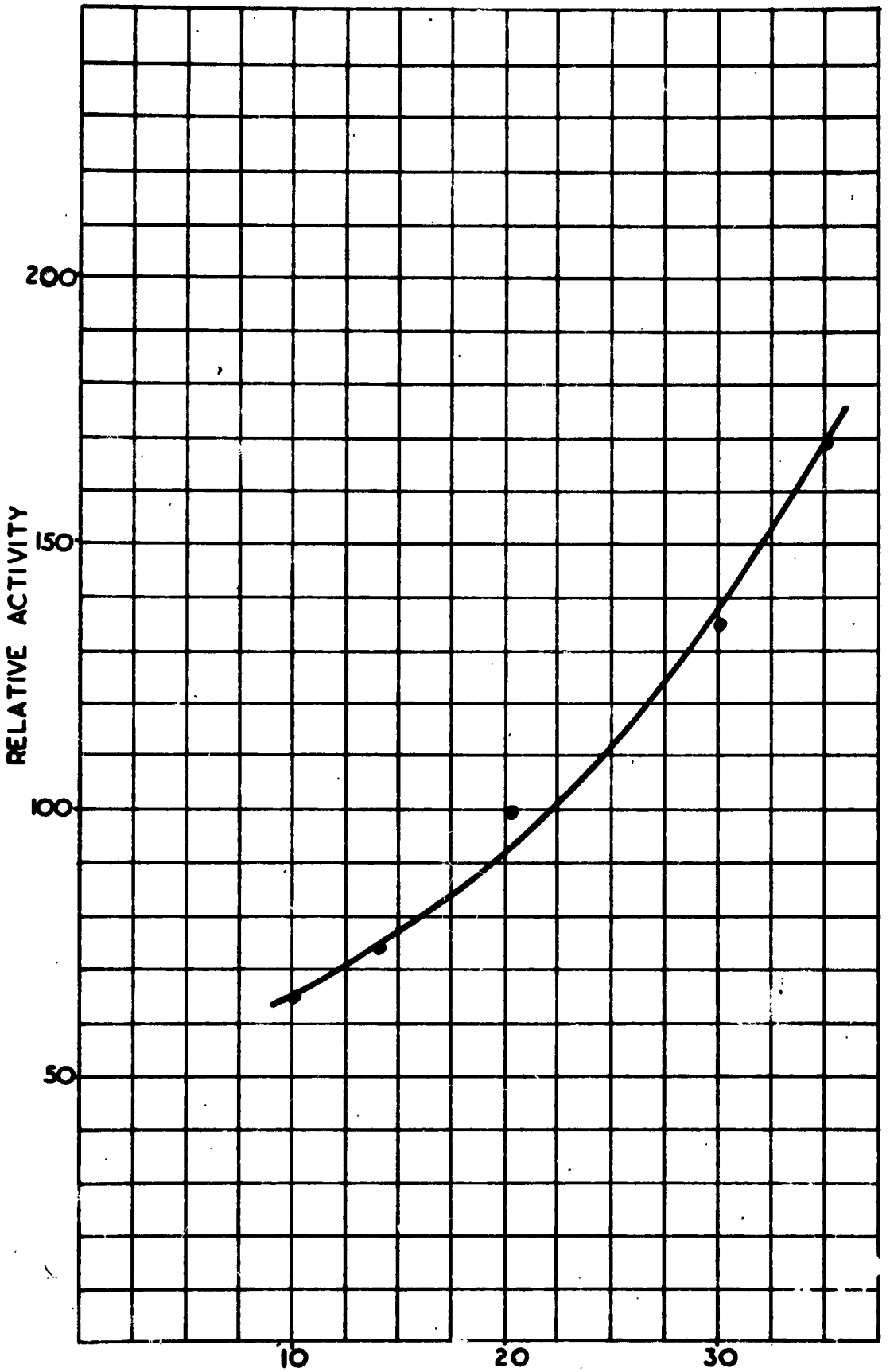


FIG 3

THE RELATIVE ACTIVITY OF WHOLE BLOOD ChE AT VARYING TEMPERATURES

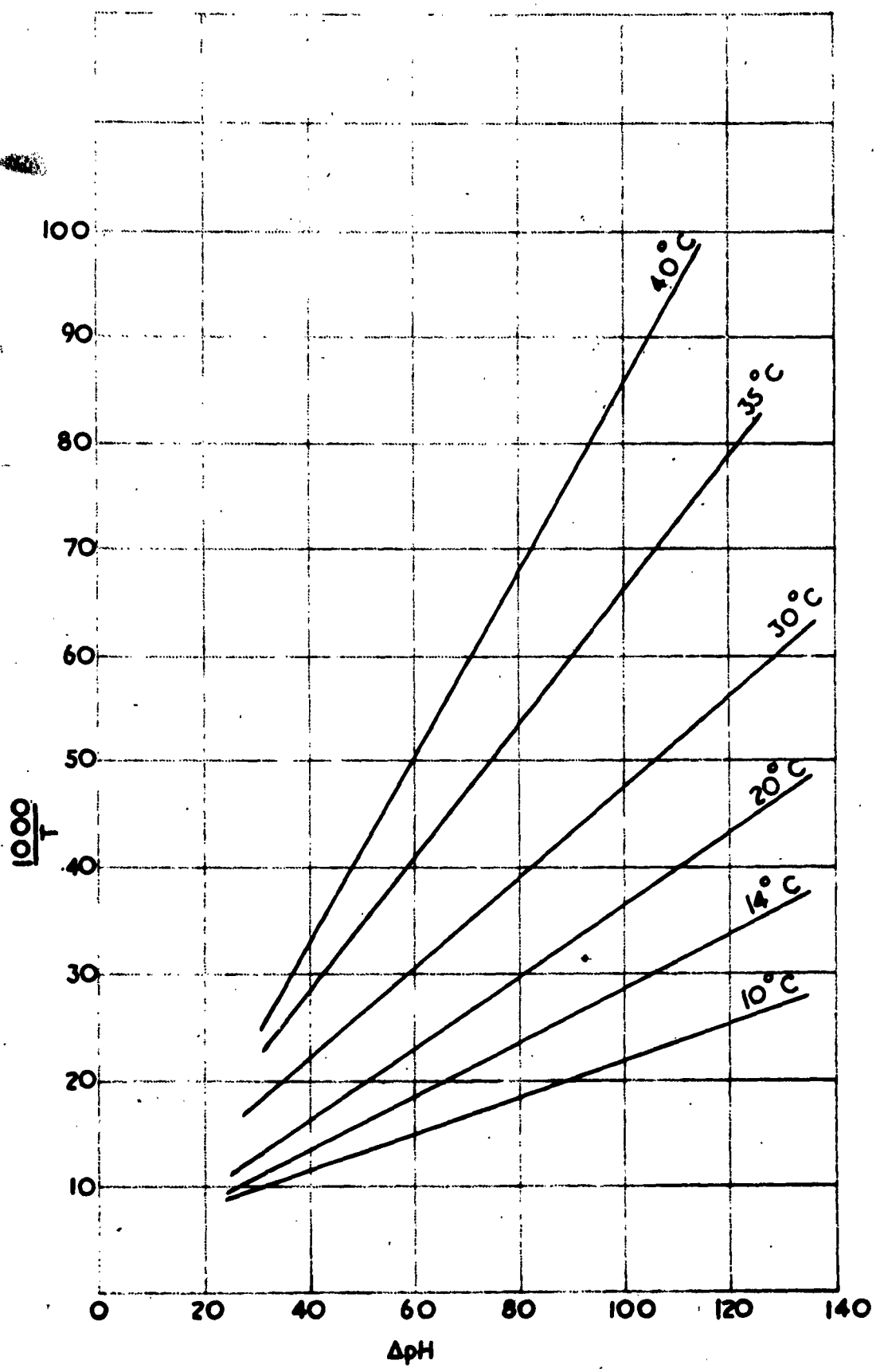


FIG. 4. THE RELATIONSHIP BETWEEN ACTIVITY A_T AND ΔpH/hr. AT VARYING TEMPERATURES.

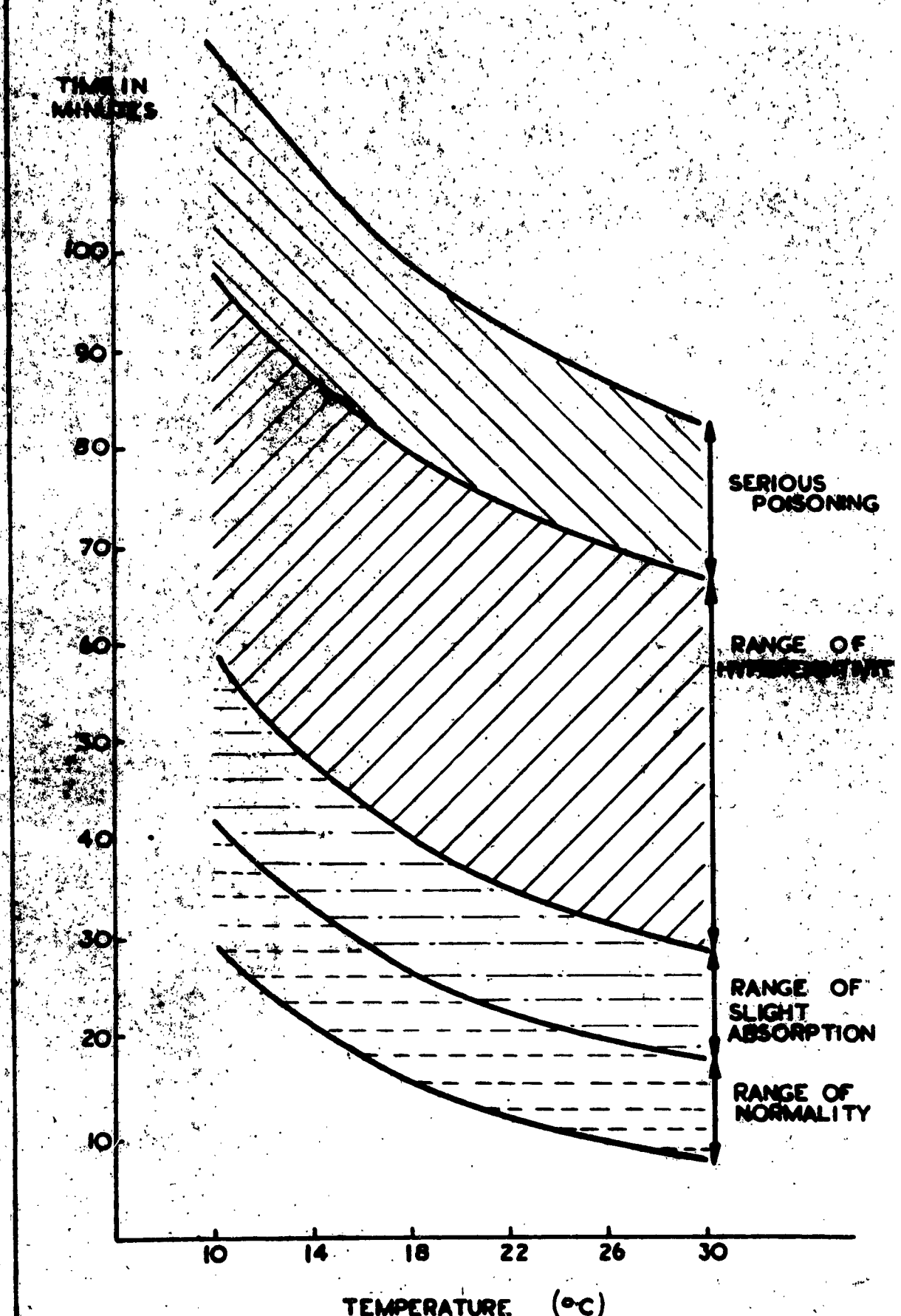


FIG 5

CHART RELATING TIME OF COLOUR CHANGE WITH TEMPERATURE

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CIRCULATION
INTERNAL

P.D.S.R.(D)
D.C.D.R.D.
A.D./C.D.R.D.
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C.D.R. Branches (2)
C.S., M.O.S. Nancekuke (2)
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R.D. Arm. 2.
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Date of Search: 29 May 2009

Record Summary: WO 189/778
Title: Field test for the assay of human whole blood cholinesterase
Availability Open Document, Open Description, Normal Closure before FOI Act: 30 years
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